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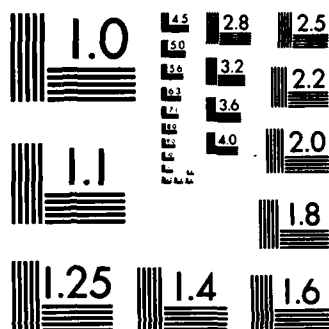   I M WARNER ET AL. 30 APR 85 EMORY/DC/TR-3
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Marine Analysis Using a Rapid Scanning

Multichannel Fluorometer

by

Isiah M. Warner, Philip B. Oldham,

Edward J. Zillioux and Gabor Patonay

Prepared for Publication

in conjunction with NATO Advanced Study Institute

on Strategies and Advanced Techniques

for Marine Pollution Studies

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April 30, 1985

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The use of a rapid scanning multichannel fluorometer capable of ship-board operation is described. The advantages of multichannel detection with an intensified linear photodiode array are discussed in reference to the continuous monitoring of oceanic phytoplankton populations by their in vivo fluorescence. The utility of the excitation-emission matrix (EEM) for fingerprinting marine algae is presented along with preliminary data acquired from unialgal cultures. Experimental data acquired at San Diego Bay, California in December '83 is presented to illustrate the utility of the instrumentation.

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MARINE ANALYSIS USING A RAPID SCANNING MULTICHANNEL FLUOROMETER

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Abstract

The use of a rapid scanning multichannel fluorometer capable of
ship-board operation is described. The advantages of multichannel
detection with an intensified linear photodiode array are discussed in
reference to the continuous monitoring of oceanic phytoplankton
populations by their in vivo fluorescence. The utility of the
excitation-emission matrix (EEM) for fingerprinting marine algae is
presented along with preliminary data acquired from unialgal cultures.
Experimental data acquired at San Diego Bay, California in December
1983 is presented to illustrate the utility of the instrumentation.

Introduction

Fluorescence detection of pigments associated with marine
phytoplankton has been used extensively for three decades. Yentsch and
Menzel (1), in 1963, introduced the use of a fluorometric method for
the selective detection of the chlorophylls and two carotenoid
pigments extracted from algae. This method provided a significant
improvement in sensitivity and selectivity over the older
spectrophotometric technique developed by Richards and Thompson (2).
Lorenzen (3), in 1966, used the inherent sensitivity of fluorescence
to continuously monitor oceanic phytoplankton populations. By
detecting the in vivo fluorescence of chlorophyll a with a simple

filter fluorometer, he was able to acheive parts-per-trillion detection in spite of an order of magnitude lower quantum efficiency for cellular chlorophyll a in solution. Numerous investigators (4-8) have continued to capitalize on the various advantages of phytoplankton monitoring by fluorescence. Unfortunately, very little has been done in exploiting the tremendous selectivity of fluorescence. Fluorescence measurement as a spectroscopic tool is fairly unique in that two wavelengths (excitation and emission) are necessary to define the detected signal. The analytical utility of two-dimensional fluorescence data (excitation and emission spectra) has been thoroughly investigated for this reason (9-13). These two dimensions of information enhance the possibility of identifying most fluorophores. Yentsch and Yentsch (14) have investigated the use of excitation spectra for gross characterization of algal classes. They introduced a fluorescence intensity ratio of two different excitation wavelengths as a discriminating parameter. However, they note in their paper that this type of investigation is difficult with the conventional scanning fluorometer and that it would be facilitated by a new instrument capable of rapid multiwavelength detection. Such a fluorometer has recently been described in the literature (15). This system is a microprocessor-controlled, multichannel fluorometer which possesses the characteristics of low detection limits, rapid data acquisition, and portability necessary for marine analysis.

Since the two-dimensional fluorescence spectrum in digital form may be represented as a conventional mathematical matrix, it is often referred to as an excitation-emission matrix (EEM). The EEM can be considered a spectral or digital image similar to a common photograph. Therefore, just as a photograph can be used for the identification of objects, so can an EEM be used in the identification of fluorescent sample. Various pattern recognition techniques (16-18) have been

useful in the discrimination of fluorescent analytes by their spectral fingerprints. Recently, a new method was developed (19) that uses Fourier-transform-based correlation for spectral matching in the frequency domain. This technique has proven to be an efficient method for the identification of single-component fluorescent samples by comparison to a library of known spectra.

This paper presents the use of a rapid scanning multichannel fluorometer for marine analysis. The various instrumental factors affecting the rapid acqusition of multiwavelength fluorescence spectra at low light levels are discussed. A preliminary investigation is described in the use of EEMs as spectral fingerprints for laboratory cultures along with two-dimensional Fourier-transform-based pattern recognition. Data acquisition and information content of field data collected at San Diego Bay are also presented.

Methods

Instrumentation

The fluorometer used in this study was a portable, multichannel instrument (PMF) (Fig. 1) that has been recently described in the literature (15). This instrument uses an intensified linear photodiode array and 1710 multichannel analyzer (Tracor Northern, Middleton, Wisconsin) for rapid scanning capabilities at low light levels. This instrument can acquire an emission spectrum over a 600 nm wavelength range in as little as 5 msec. An EEM is acquired by sequentially capturing emission spectra at various excitation wavelengths. An AC stabilized (20) 150 watt DC xenon arc lamp (Ealing Corp., South Natick, Maine) is used as the excitation source. The individual excitation wavelengths are selected by a circular variable filter wheel (Optical Coating Laboratory, Santa Rosa , California)
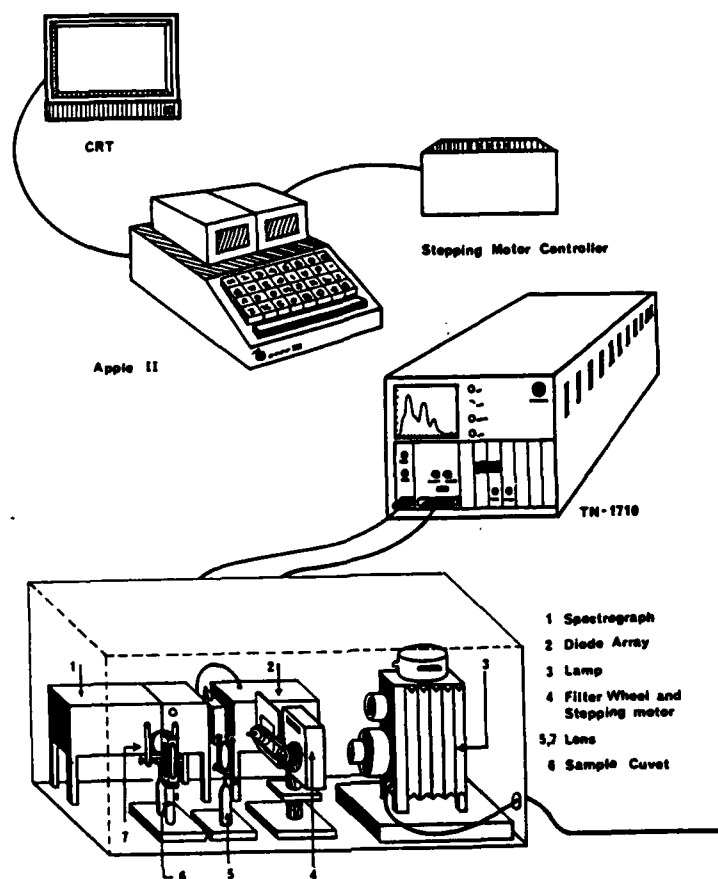
Fig. 1. Diagram of the portable multichannel fluorometer (PMF).

that is driven by a stepping motor. The filter wheel is a continuously variable interference filter which transmits visible light from approximately 350 nm to 750 nm with about a 15 nm bandpass. The individual emission spectra are acquired by the photodiode array and then stored for future access by the controlling Apple II+ microcomputer. A 10 Mbyte Winchester drive hard disk has been added. This addition extends the mass storage capacity and cuts the data access time in half. A sequential scan module also has been added to allow the 1710/ photodiode array to acquire up to 64 emission spectra at reduced resolution in a triggered mode. Since fluorescence spectra are generally broad in nature, the reduced spectral resolution does not adversely affect the data and allows for more efficient data

acquisition and handling. In this study, 128 data point emission spectra were acquired at 32 different excitation wavelengths. This is later averaged down to a 32 X 32 matrix in software allowing up to 186 complete EEMs to be stored on the hard disk. The triggered mode of spectral acquisition also enables the software to more reliably and efficiently coordinate data handling operations.

Experimental

The algae used in the laboratory studies were selected to provide a representative sampling of the various marine classes. A listing of the algal species investigated is provided in Table I. Listings

Table I. Laboratory algae collection.

Class	Species	Source[*]	Media
Chlorophyceae	Chlorella vulgaris	1	ASP 6
Chlorophyceae	Dunaliela salina	2	ASP 6
Prasinophyceae	Tetraselmis sp.	1	ASP 6
Bacillariophyceae	Bellerochea horologicalis	1	ASP 6
Bacillariophyceae	Skeletonema costatum	2	ASP 6
Dinophyceae	Amphidinium carterae	3	ASP 6
Cyanophyceae	Spirulina major	3	ASP 6
Prymnesiophyceae	Prymnesium parvum	1	ASP 6
Prymnesiophyceae	Coccolithophora sp.	2	Soil-Seawater

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 3) Carolina Biological Supply Company, Burlington, North Carolina

of the respective algal classifications, culture media, and sources are also given. All of the species listed except Coccolithophora sp. were grown in our laboratory. A 12 hr./12 hr. light/dark cycle was used for all of the species except Spirulina major which was kept on the shelf in low light. The medium used was Provasoli's ASP 6 (21) artificial seawater adjusted to a pH of 7.6 with concentrated HCL. The temperature was not well regulated but was maintained between 16 °C and 25° C. Individual samples were withdrawn from the culture tubes with

disposable pipets and injected into a standard 1 cm quartz fluorescence cuvet. If the cultures were too dense the samples were diluted with fresh media in order to prevent inner filter effects. The EEMs of media blanks were subtracted from the culture EEMs to correct for any fluorescence and/or scattering from the media itself. Scan times for spectral acqusition by the photodiode array were selected so as to provide an adequate signal-to-noise ratio. The typical spectra were acquired by signal averaging 30 emission scans of 25 msec. each.

The field data presented were gathered in San Diego Bay in December 1983. Water was pumped continuously from various depths to a small houseboat that was docked just off shore. Fluorescence data were acquired by the multichannel fluorometer equipped with a flow cell for sample through-put. The spectral acquisition parameters for these data were similar to those of the laboratory spectra except a scan time of 1 sec. was used. Fluorescence data from a Turner Model III fluorometer were recorded for comparison. Ancillary data such as water temperature, depth, pH, salinity, and time were acquired along with the fluorescence data.

Results and Discussion

Representative EEMs of the 5 algal classes sampled in the laboratory are pictured in Fig. 2. One can immediately recognize that most of the distinctive information is contained in the excitation (y-axis) spectrum. This is true for all but the blue-green species, which exhibit shorter wavelength emission due to the presence of the phycobilin pigments. In all of the other spectra, chlorophyll a is the primary emitter due to the transfer of energy from the secondary pigments (other chlorophylls and carotenoids) to chlorophyll a. Generally, there are three areas of the EEM that appear useful in characterizing an algal sample. These pertain to the 3 major pigment
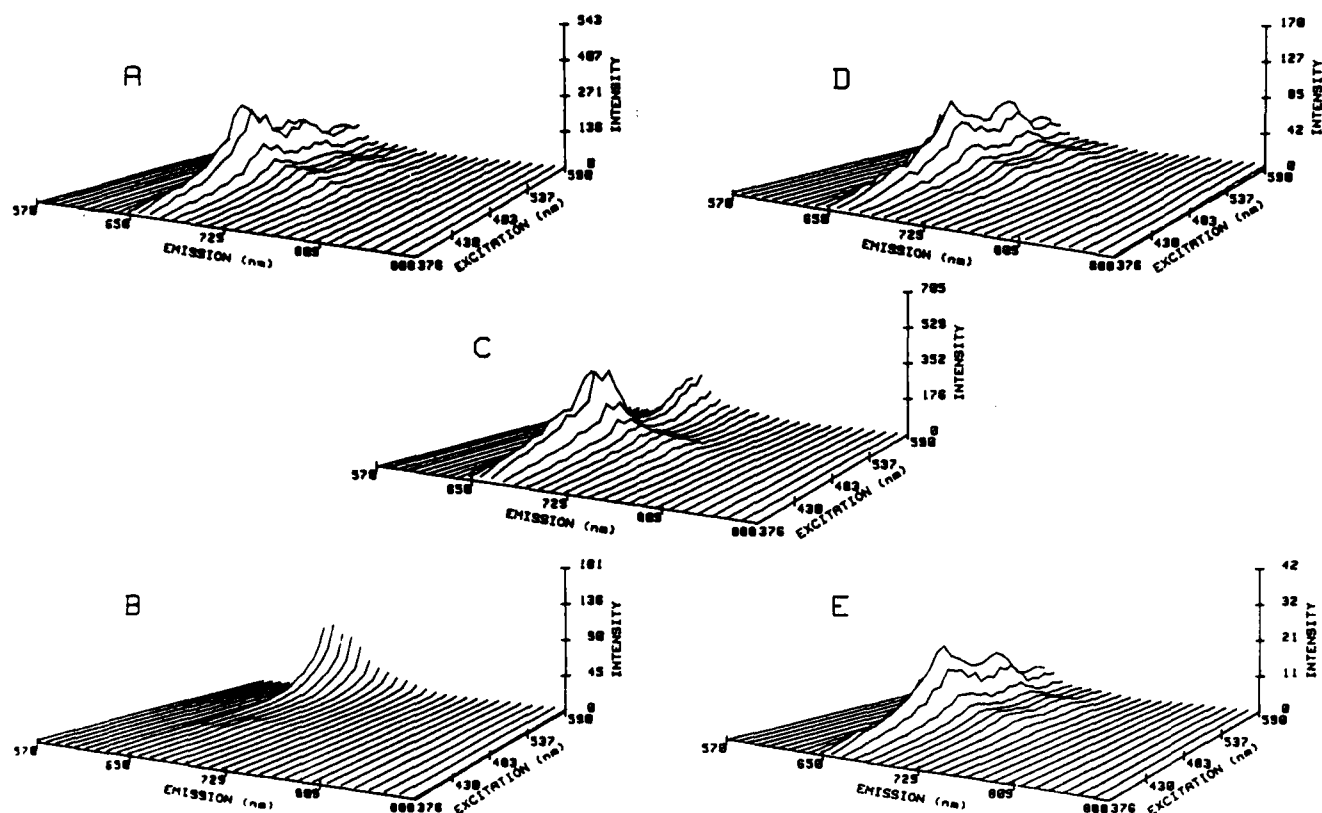
Fig. 2. EEMs of 5 algal classes. A) Prymnesiophyceae; B) Cyanophyceae;
C) Chlorophyceae; D) Bacillariophyceae; E) Dinophyceae.

systems which we will refer to as the chlorophylls, carotenoids, and

phycobilins. Fig. 3 indicates the important areas of the EEM with the

responsible pigment system. The carotenoids do not exhibit natural

fluorescence; however, their presence is easily detected in the EEM

due to the transfer of excitation energy to chlorophyll a. As

previously noted, many of these spectra are easily distinquished by

sight. The greens and blue-greens differ greatly from the browns

(diatoms, dinoflagellates, and golden-browns). However, it is more

difficult to distinquish between the various browns since all of them

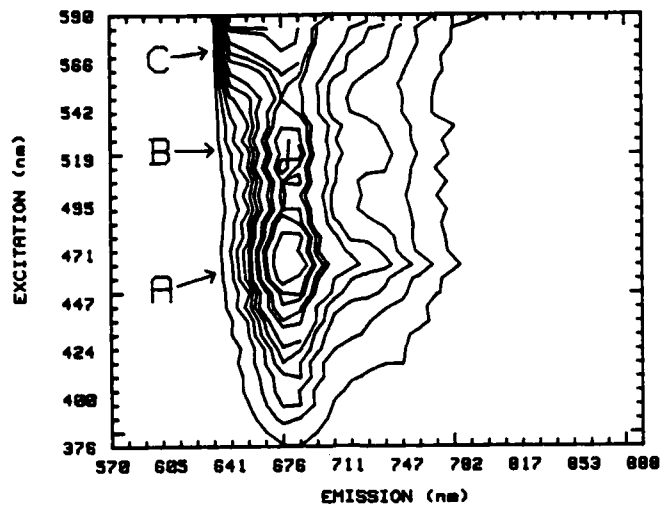contain a carotenoid complex. The attempt by Yentsch and Yentsch (14)

Fig. 3. Spectral areas of interest. A) chlorophylls; B) carotenoids; C) phycobilins.

to use the excitation ratio was not successful in making such a distinction. In fact, our preliminary investigations suggest that the use of wavelength ratios is very ambiquous. The ratios are dependent on the species sampled, the physiological condition of the sample, and the ability to properly correct for fluorescence background. Based upon this and the pioneering work that has been done in two-dimensional pattern recognition, a more sophisticated approach seemed appropriate.

A group of 9 unialgal species (6 classes) was selected to investigate the ability of two-dimensional Fourier transform based pattern recognition to distinquish between algal groups. Replicate EEMs were recorded for the 9 cultures. The replicates were not of the exact same sample but were separate samples taken from the same culture. One set of the EEMs was selected arbitrarily to comprise the library of "standard" EEMs. The other set was used as "unknowns" to determine the effectiveness of the computer software in correctly matching the replicate spectra. These spectra were, of course, not true unknowns in that their origins were known by the investigator. The results of the spectral matching according to 3 different parameters (A, B, and C) are listed in Table II. It is important to

recognize that the correct match occurred on the first hit in every case. Even more important was the discovery that the hits tended to group by taxonomic relationship. These preliminary results are certainly encouraging enough to suggest that such a pattern recognition system may be capable of a higher level of differentiation than just gross characterization.

Table II. Results of spectral matching.

| | Hit # | | |
Specie	A	B	C
Chlorella vulgaris	1	1	1
Dunaliela salina	1	1	1
Tetraselmis sp.	1	1	1
Spirulina major	1	1	1
Skeletonema costatum	1	1	1
Bellerochea horologicalis	1	1	3
Amphidinium carterae	1	1	1
Prymnesium parvum	1	1	2
Coccolithophora sp.	1	1	1

The next step is to apply such a technique to natural populations. A representative EEM acquired from San Diego Bay is pictured in Fig. 4. Unfortunately, these data were taken before
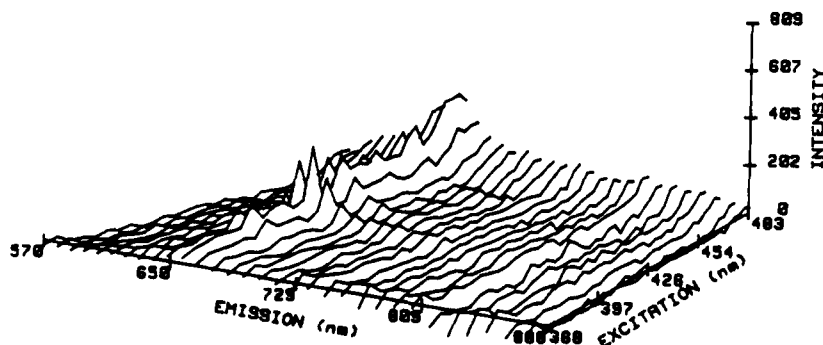

Fig. 4. Typical EEM from San Diego Bay.

sufficient laboratory cultures could be investigated and the excitation wavelength range is not quite broad enough to perform

adequate pattern recognition. However, it does illustrate the ability

of the multichannel fluorometer to acquire EEM data in the field. It

is also apparent from the presence of the carotenoid complex that some

brown phytoplankton species, possibly diatoms, predominate the bay

water.

There are other experimental parameters that also can be

exploited due to the rapid data acquisition capabilities of this

fluorometer. Fig. 5 illustrates how elapsed time can replace

excitation wavelength in the two-dimensional format to form what may

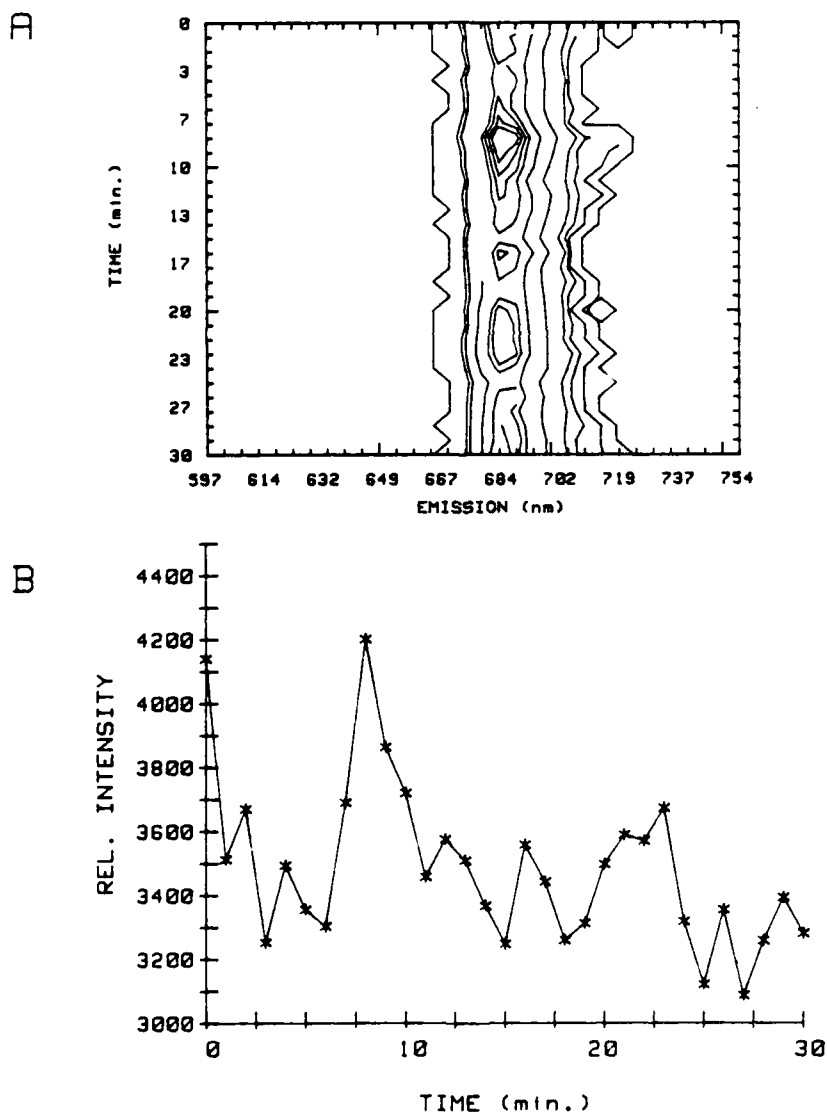be called a time-emission matrix (TEM). Since there is no mechanical

Fig. 5. A) Contour plot of a typical TEM from San Diego Bay;
B) One-dimensional data taken from the TEM; λ_{ex} = 430 nm;
λ_{em} = 700 nm.

scanning associated with the acquisition of emission spectra, the time resolution can be as short as seconds. This is one way of detecting changes in sample composition and/or concentration with respect to relative position.

A third example of the type of matrix formatted data that can be collected by this instrument is the depth-emission matrix (DEM) as shown in Fig. 6. Since there was no means of accurately or easily
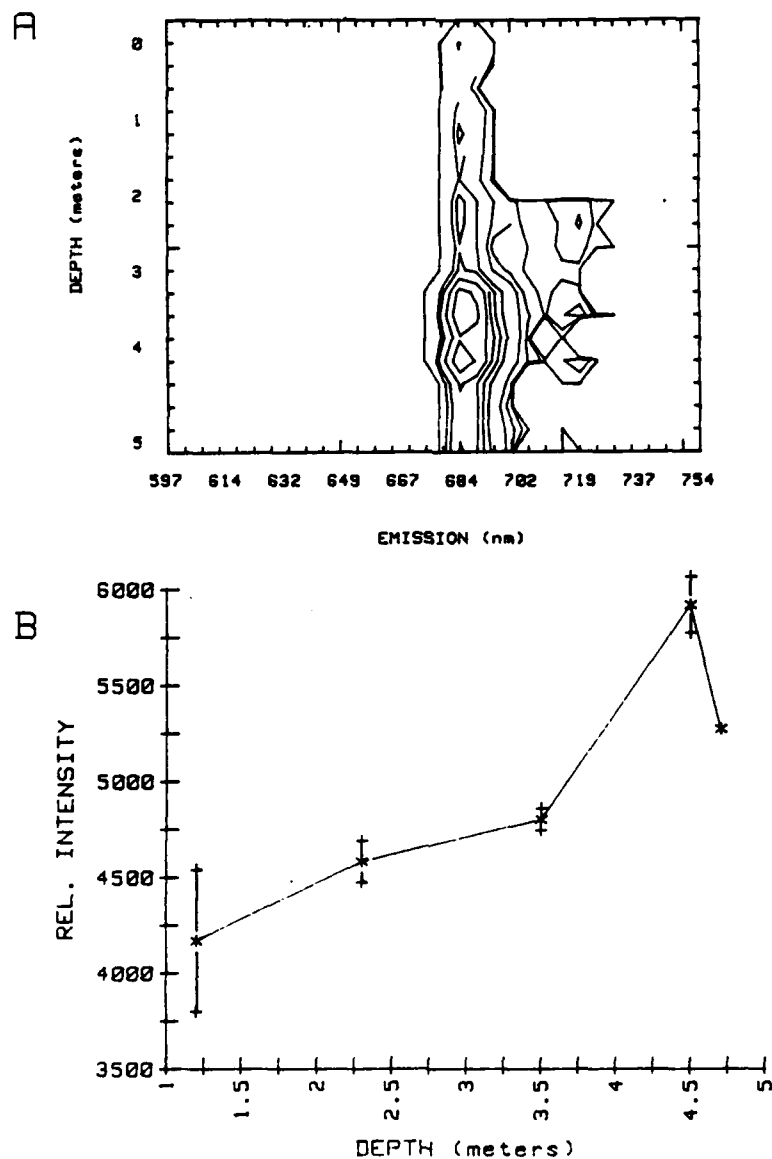

Fig. 6. A) Contour plot of a simulated DEM from San Diego Bay;
B) One-dimensional depth vs. fluorescence intensity;
λ_{ex}= 430 nm; λ_{em} = 700 nm.

varying the probe depth, the data illustrated in Fig. 6(B) was acquired by taking 4 emission spectra at 5 different depths for a

total of 20 emission spectra. The error bars shown signify the range of values within 1 standard deviation of the mean. Five points from 5 different depths are not sufficient to illustrate a DEM, but if all 20 emission spectra had been acquired at different depths we could plot a DEM as shown in Fig. 6(A). It is a potentially powerful method of determining the phytoplankton variability within the water column.

The support data collected from San Diego Bay for comparison are tabulated below where Fmax is the maximum fluorescence intensity from the PMF, C is the fluorescence signal recorded by the Turner fluorometer, and %T is the turbitity recorded by a photometer. The

Table III. Data collected from San Diego Bay.

#	Fmax	C	%T	Depth	Tide	Time
1	5495	-	-	-	High	0943
2	3095	-	-	-	Low	1610
3	2784	-	-	-	Low	1640
4	3864	4.0	18	1.5	High	1155
5	4492	10.0	22	1.5	High	1230
6	5668	5.5	22	1.5	High	1345
7	2642	4.8	38	2.0	Low	1630
8	2464	4.7	26	2.0	Low	1810
9	313ɔ	6.5	28	2.0	Low	2030
10	2565	3.6	19	1.8	High	1005
11	3464	3.5	15	1.8	High	1025
12	3935	5.1	33	2.5	High	1155
13	7590	4.5	16	1.5	High	1120
14	13655	5.2	22	1.5	High	1310
15	15852	6.0	40	4.6	Low	1555
16	10846	4.8	33	5.8	High	1115

chlorophyll fluorescence measured by the Turner fluorometer is especially important since it should be comparable to the PMF measurment. However, the two methods did not compare very favorably. These differences could be due to improper calibration of the Turner fluorometer or to a difference in the linear dynamic ranges of the two instruments. Further investigations are planned to compare the two techniques more accurately. The other data in Table III is generally useful in determining the daily variation of phytoplankton activity, as well as, locating the most productive regions of the water column.

Conclusions

The data presented indicate a potential for characterizing laboratory phytoplankton cultures and also the ability to perform similarly on-board ship. Although not expressly covered in this paper, two-dimensional fluorescence also has excellent quantitative capabilities. Further studies are examining the utility of the multichannel fluorometer for the identification and quantitation of analytes in the marine environment. Expansion of the library of standard spectra is a high priority at this time. It is hoped that eventually the spectral library will contain representative spectra of the majority of commonly occurring marine phytoplankton. We plan to include in this work a study of the potential for fingerprinting mixed populations. The development of an automatic method of analyte preconcentration so that EEMs taken in the field might have better signal-to-noise characteristics is also being investigated. This would improve the pattern recognition reliability and effectively lower the detection limits.

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